

Mechanisms of population regulation in the mutualism between *Breynia vitis-idaea* (Euphorbiaceae) and *Epicephala vitisidaea* (Lepidoptera, Gracillariidae)

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Abstract: 【Aim】 In order to study how *Breynia vitis-idaea* controls the population of *Epicephala vitisidaea*. 【Methods】 The phenology of *B. vitis-idaea* and the biology of *E. vitisidaea* were recorded. The flower-visiting *Epicephala* moth species were identified by dissecting their genitalia. For calculating larval survivorship, the number of larvae inside the fruit, the number of exit holes and oviposition scars on the fruit were counted by dissecting the stalked and non-stalked fruits in summer and late autumn. Finally, the proportion of stalked and non-stalked fruits in summer and late autumn was calculated. 【Results】 In Xiamen, Fujian Province, *B. vitis-idaea* plants have five peak fruiting stages annually and the flower-visiting *Epicephala* moths have five generations each year. The species of moth was identified as *E. vitisidaea* by contrasting its genitalia. *E. vitisidaea* larvae need to consume 2–4 seeds to develop into mature larvae. *B. vitis-idaea* could produce two types of fruits, stalked and non-stalked. The larval survivorship of *E. vitisidaea* was obviously higher in non-stalked fruits than in stalked fruits as well as in late autumn than in summer. Meanwhile, the proportion of non-stalked fruits of *B. vitis-idaea* was obviously higher in late autumn (82.04%) than in summer (31.53%). 【Conclusion】 This study revealed the mechanism by which mutualistic interactions are more stable. *B. vitis-idaea* can control the larval survivorship of *E. vitisidaea* by producing the stalk in base of fruits. By seasonally regulating the ratio of stalked and non-stalked fruits, *Breynia* plants prevent seed overconsumption in summer and ensure *Epicephala* survival through the winter. The auto-regulating mechanism of *B. vitis-idaea* plays an important role in keeping stability of mutualistic system between *B. vitis-idaea* and *E. vitisidaea*.

Key words: *Breynia vitis-idaea*; *Epicephala vitisidaea*; fruit stalk; larval survivorship; obligate pollination mutualism; regulation mechanism

1 INTRODUCTION

Mutualisms are reciprocally beneficial interspecific interactions, which are viewed as balanced antagonistic interactions that nonetheless provide net benefits to each mutualist (Axelrod and Harnilton, 1981; Bull and Rice, 1991). They are found at all levels of biological organization and are widely appreciated for their fundamental importance in the evolution and maintenance of biodiversity (Herre *et al.*, 1999; Kawakita and Kato, 2006). There are, however, clear conflicts of interests in mutualisms between plants and seed-parasitic pollinators. Plants depend on the pollination of flower-visiting insects but must limit the seed consumption by the larvae of

these insects. In the mutualism between figs and fig wasps, for example, figs limit the number of eggs laid by fig wasps by varying the length of flower styles (Cook and Rasplus, 2003). In the mutualisms between yucca trees and yucca moths, and between *Glochidion* trees and *Epicephala* moths, plants selectively abscise flowers with high egg loads to prevent excessive seed destruction by moth larvae (Pellmyr and Huth, 1994; Goto *et al.*, 2010).

Kawakita and Kato (2004) found that an *Epicephala* moth obligately pollinates *B. vitis-idaea*. They found that single *Epicephala* larva consumed a fraction of seeds within developing fruit in *B. vitis-idaea* and some of the fruits were left untouched. For many of these untouched fruits with indication of moth oviposition, they suggested that egg/larval mortality of

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Epicephala moths is an important factor assuring seed set in these plants. Svensson *et al.* (2010) showed that system-specific chemistry is not necessary for efficient host location by exclusive pollinators in mutualism between *B. vitis-idaea* and its host-specific *Epicephala* pollinator. Zhang *et al.* (2012b) found that different structures in the female ovipositor and oviposition mode caused different population of co-pollinators: *E. mirivalvata* and *E. lativalvaris*.

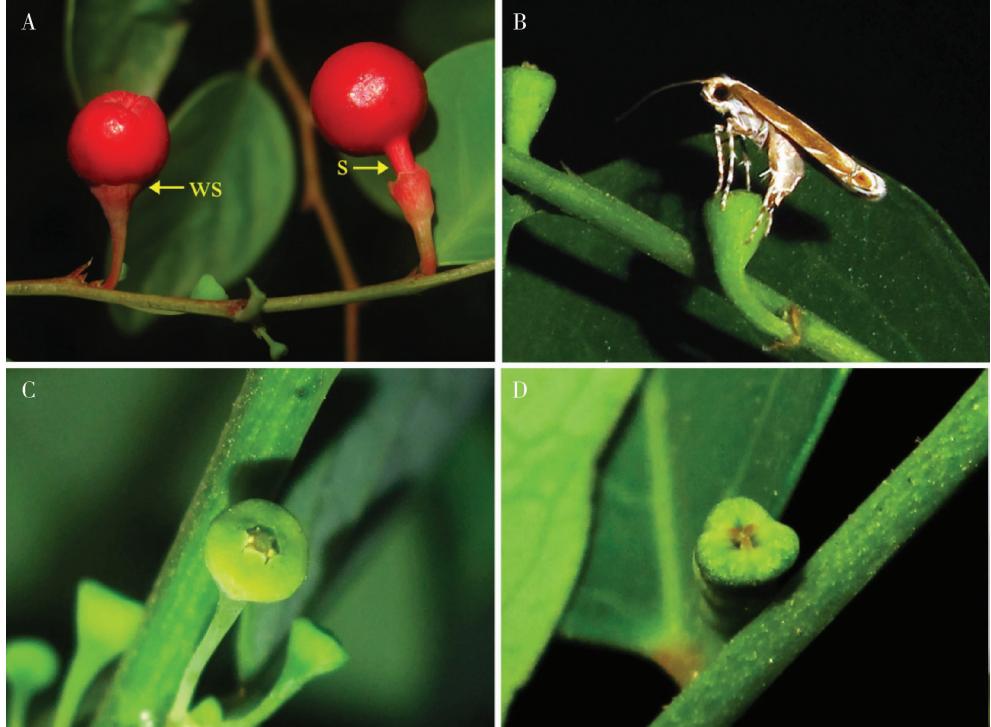


Fig. 1 Morphology of *Breynia vitis-idaea*

A: Fruits of two types on one branch; a fruit without a stalk (left), and a fruit with a stalk (right); B: An *E. vitisidaea* moth laying an egg; C: Male flower; D: Female flower. WS: Non-stalked; S: Stalk.

2 MATERIALS AND METHODS

2.1 Studied plant

Breynia vitis-idaea (Burm. f.) Fischer is an evergreen and perennial species of Phyllanthaceae. It is a monoecious shrub that can reach 1.5–5.0 m in height. It grows in montane scrubs at 150–1 000 m in Fujian Province and is widely distributed in China (Guangdong, Guangxi, Guizhou, Taiwan, Yunnan), across southeastern Asia and the Indian subcontinent (Li, 1994; Li and Gilbert, 2008). Sepals of both male and female flowers are fused in an inverted mitriform (Fig. 1: C, D), which makes it hard to be touched by other flower visitors except *Epicephala* moths. Stamens and stigmas are concealed within the calyx, making them difficult to be touched by common flower visitors. Upon maturation, the calyxes of male flowers dehisce to

In Fujian Province, we found that *B. vitis-idaea* had two types of fruits, stalked and non-stalked (Fig. 1: A), and confirmed that *E. vitisidaea* moth was the obligate pollinator of *B. vitis-idaea* plant. Here we focused on the two types of fruits of *B. vitis-idaea* to investigate the phenology, observed the life history, oviposition behavior and larval survivorship of *E. vitisidaea*. Finally, we answered how the *B. vitis-idaea* controlled the population of *E. vitisidaea* through bearing stalked and non-stalked fruits.

form a small pit at the apex. Fruits are spherical and red to red-purple in color. According to Li and Gilbert (2008), the plants with stalked fruits belong to *B. officinalis*, and the plants with non-stalked fruits belong to *B. vitis-idaea*. However, Li (1994) considered *B. officinalis* as a synonym of *B. vitis-idaea*. In this study, we firstly found that *B. vitis-idaea* with two fruit types coexist on the same individual plant (Fig. 1: A). Here we call these fruits “stalked” or “non-stalked”. The stalked fruit is elevated a short distance above the calyx, whereas the non-stalked fruit clings to the top of the calyx. So we regard the presence or absence of stalk as well as the variation of the leaf shape as intraspecific variations, and confirm that *B. officinalis* is a synonym of *B. vitis-idaea*. As the host plant, *B. vitis-idaea* provides food that the *E. vitisidaea* larvae use to grow and develop.

2.2 Studied insect

Epicephala vitisidaea Li, Wang & Zhang, is a pollinating moth species of Gracillariidae in the order Lepidoptera (Zhang et al., 2012a). Proboscises of the pollinating adult female have cilia for collecting and transporting pollen grains. These cilia are absent from the non-pollinating adult males. *E. vitisidaea* adults have a grayish brown or yellowish brown forewing (Fig. 1: B; Fig. 2: A) with three parallel white stripes that extend obliquely outward from the basal $1/3$, $3/5$, and $3/4$ of the costal margin. They also have a broad creamy white band on the dorsal margin. The costa is nearly parallel dorsoventrally, with long dense ventral setae in male genitalia. Mature larvae are $5.0 - 6.5$ mm in length, with a

black head, a dark red thorax, and a white abdomen. The middle part of each abdominal segment is red or dark red (Fig. 2: B). The body is sparsely covered with setae. As the host plant, *B. vitis-idaea* provides food that the *E. vitisidaea* larvae use to grow and develop. Larvae feed on *B. vitis-idaea* seeds. Larvae exit the fruit when they have developed to maturity and pupate on the leaves. Cocoons are $6.0 - 7.5$ mm in length, white, elliptical in shape, with some white grains attached to the surface (Fig. 2: C). Pupae are $4.0 - 5.0$ mm in length, fusiform, and pale yellow during early pupal stages (Fig. 2: D). They gradually become grayish brown before eclosion.

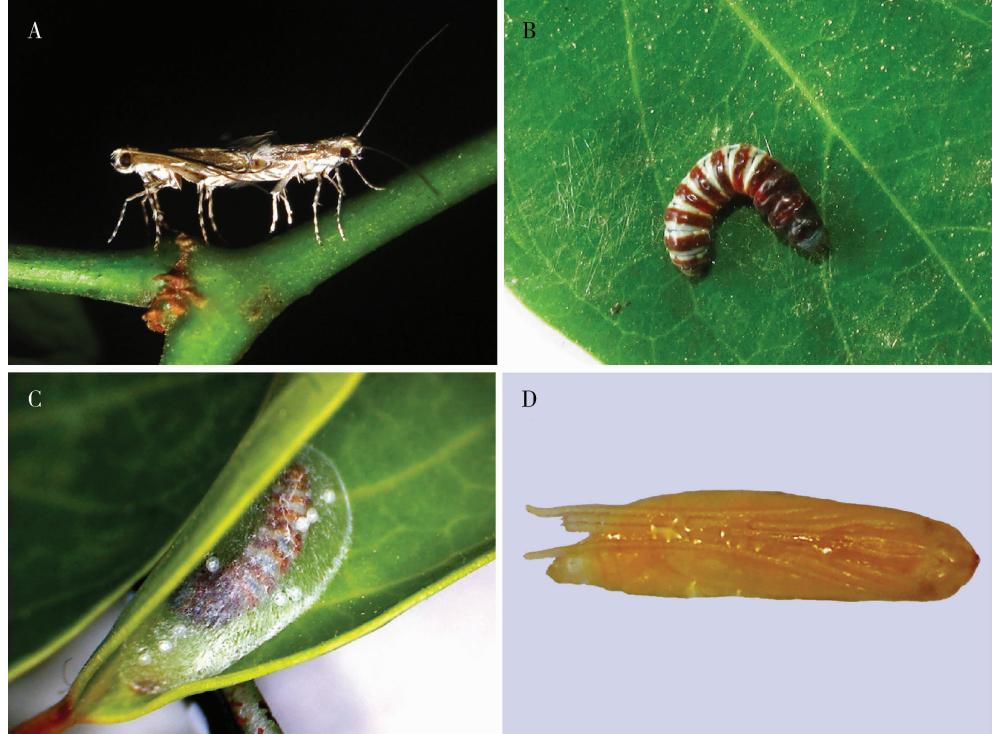


Fig. 2 Life history of *Epicephala vitisidaea*

A: Coupling moths; B: A mature larva that has exited from a fruit and is ready to pupate on the host leaf; C: A pupal cocoon woven by a mature larva on a host leaf; D: A pupa that has been stripped from a cocoon.

2.3 Determination of mutualism systems

We tracked the development of *B. vitis-idaea* flowers and fruits in Xiamen, Fujian Province from June 2010 to September 2011 for a phenological study and collected fruits when they had matured. To ascertain the oviposition behavior of *E. vitisidaea*, we dissected 109 pollinated female flowers that were collected from June 2010 to September 2011 at Xiamen University, Jinbang Park, Hongshan Park and Gulang Island in Xiamen, Fujian Province. We also dissected the genitalia of flower-visiting *Epicephala* moths to identify the species. To determine the impact of the fruit stalk on larval

survivorship, we collected and dissected stalked fruits of different developmental stages, specifically asking whether the eggs had yielded viable larvae. We dissected 532 stalked and 397 non-stalked mature fruits from 15 individual *B. vitis-idaea* plants in summer (June to August) and late autumn (November). We counted the number of oviposition scars on the fruit to confirm the number of eggs and counted both the number of larvae inside the fruit and the number of exit holes to calculate larval survivorship. To calculate emergence rates, we reared larvae (see below) and counted the number of pupae and the number of adults that survived. The

emergence rate equaled the number of adults divided by the number of pupae.

Mature fruits were collected randomly from 15 individual plants every fruiting period (4 in Xiamen University, 3 in Jinbang Park, 5 in Hongshan Park, and 3 in Gulang Island in Xiamen, Fujian Province) and placed in transparent plastic cylindrical boxes ($8.5\text{ cm} \times 12\text{ cm}$) to calculate the proportion of stalked and non-stalked fruits (the proportion of stalked or non-stalked fruits = the number of stalked or non-stalked fruits / the number of all sampled fruits). We reared *E. vitisidaea* larvae and recorded how and when the larvae exited the fruit, pupated, and emerged through field and laboratory observations. Pupae were kept in transparent vitreous cylindrical boxes ($2.7\text{ cm} \times 7.2\text{ cm}$), which were put in 40% – 60% humidity conditions.

2.4 Statistical analyses

Statistical analyses were performed with SPSS 20 (SPSS, Chicago). Different seasons and fruit types were analyzed using a two-way analysis of variance (GLM statistical model). Differences between the means were compared using Duncan's multiple-range tests at $P < 0.05$.

Table 1 Life history of *Epicephala vitisidaea* in Xiamen, Fujian Province

Generation	Apr.			May			Jun.			Jul.			Aug.			Sep.			Oct.			Nov.			Dec.			Jan.										
	F	M	L	F	M	L	F	M	L	F	M	L	F	M	L	F	M	L	F	M	L	F	M	L	F	M	L											
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4th	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–					
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5th	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)	(□)				
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

● : Egg; – : Larva; □ : Pupa; + : Adult; (□) : Overwintering pupa; F: First ten days; M: Middle ten days; L: Last ten days.

of *B. vitis-idaea* needed 10 – 25 d to develop to mature flowers from buds, and pollinated female flowers needed 20 – 25 d to develop to mature fruits. The non-pollinated female flowers could develop without pollen grains, and then withered 10 d later. All the seeds in withered flowers were infertile.

3 RESULTS

3.1 Life history of *E. vitisidaea* and phenology of *B. vitis-idaea*

By dissecting the genitalia of flower-visiting *Epicephala* moths, we determined that the pollinators of *B. vitis-idaea* were *E. vitisidaea* moths. The studied insect and voucher plant specimens are deposited in the Insect Collection of Nankai University (NKUM). Together these species formed a one-to-one obligate pollination mutual relationship. In Fujian, *B. vitis-idaea* had five peak fruiting stages annually. The first generation of male and female flowers began to bud from late March to early April, reaching the peak flowering stage in late April. The following peak flowering stages happened in early June, middle July, late August and early October. *E. vitisidaea* had five generations correspondingly, lasting from the 1st generation in middle April to the 5th generation in early November, with the overwintering generation emerging in mid-April. Mature larvae of the overwintering generation exited during early January and overwintered as pupae (Table 1). The flowers

3.2 Biology of *E. vitisidaea*

E. vitisidaea larvae must consume 2 – 4 seeds (from stalked or non-stalked fruits) to develop into mature larvae according to our study. Each mature larva gnawed a round hole in the fruit wall to exit. They then produced a cocoon and pupated on the

leaves of the host or a nearby plant. The pupal stage lasted 5–8 d. Emerged adults mated on leaves of the host or a nearby plant. Adult females ($n = 37$) actively collected pollen grains from male flowers (Fig. 3: A) to pollinate female flowers (Fig. 3: B) and then oviposited on these flowers. *E. vitisidaea* female moths may continuously pollinate 2–3 female flowers from bottom base to top end along the same

branch. The female moths deposited pollens on the stigma by repeatedly rubbing the outspread tip of the proboscis against stigma (Fig. 3: B), and laid eggs subsequently (Fig. 1: B). We also observed some female moths repeating this oviposition behavior on the same flower, but they never repeated it more than three times. *E. vitisidaea* adults survived for 3–5 d. *E. vitisidaea* is an obligate pollinator of *B. vitis-idaea*.

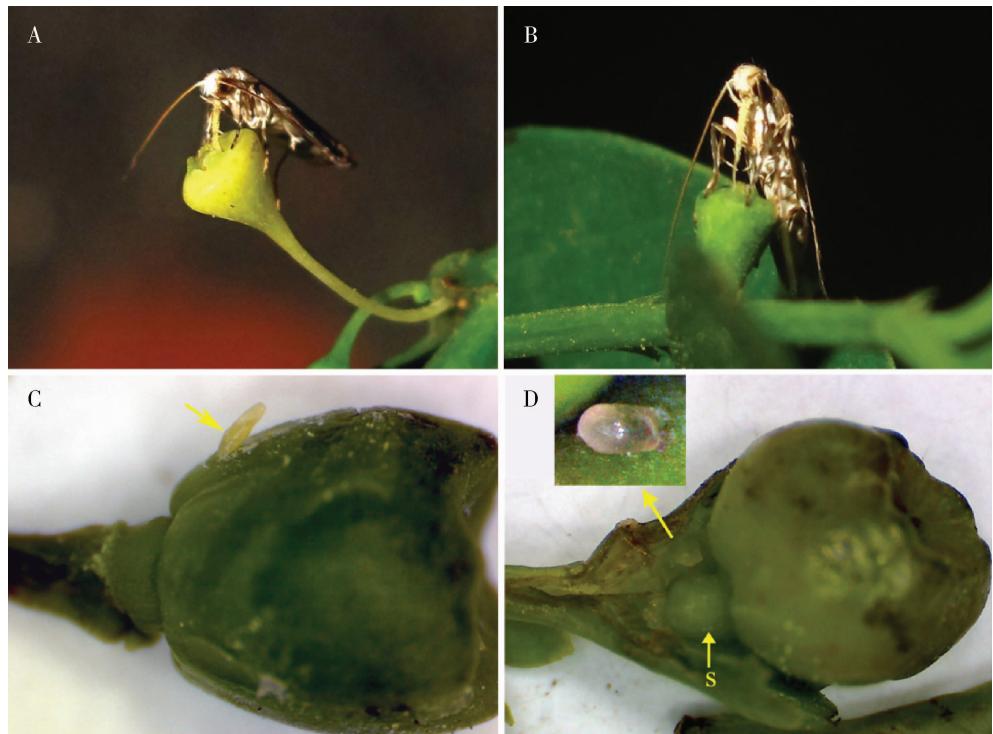


Fig. 3 Behavior of *Epicephala vitisidaea* moths

A: Female moth collecting pollen grains from a male flower; B: Female moth pollinating a female flower; C: An egg laid on a non-stalked fruit of *B. vitis-idaea*; D: An egg laid on a stalked fruit of *B. vitis-idaea*. S: Stalk.

3.3 Number of *E. vitisidaea* eggs deposited on *B. vitis-idaea* female flowers

By dissecting female flowers ($n = 109$), we determined that all pollinated female flowers of *B. vitis-idaea* loaded *E. vitisidaea* eggs. This agreed with our field observation that all *Epicephala* individuals oviposited after pollinating flowers at night (Fig. 1: B). Most pollinated female flowers (80.73%) only had one egg, and the rest had 2–4 eggs (Fig. 4). *E. vitisidaea* laid eggs between the ovary and the calyx of *B. vitis-idaea* female flowers (Fig. 1: B). The eggs could be deposited on the upper (Fig. 3: C), middle, or lower part of the ovary. The female flower buds did not have stalks and the stalks developed before the eggs hatched (Fig. 3: D).

3.4 Statistics of *E. vitisidaea* eggs and larval survivorship

We calculated the number of eggs associated

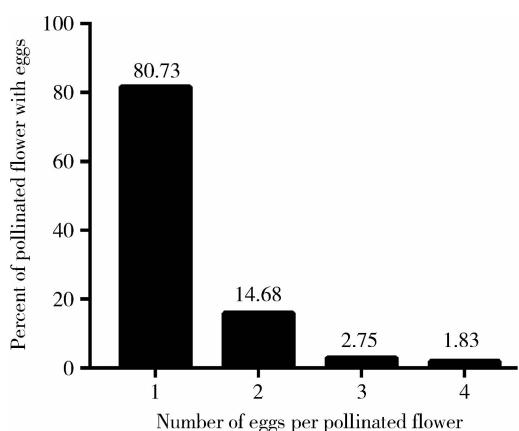


Fig. 4 Percent of *Epicephala vitisidaea* eggs per pollinated female flower of *Breynia vitis-idaea*

with each fruit by adding the number of larvae and the number of exit holes. Larval survival rates in stalked fruits (27.31%, $n = 495$ in summer; 29.73%, $n = 37$ in late autumn) were lower than

those in non-stalked fruits (63.30%, n = 228 in summer; 72.69%, n = 169 in late autumn) (Table 2). Moth larvae were less likely to survive on stalked fruit, so this type of fruit limited seed consumption. During the summer, the proportion of stalked fruit was high, which promoted seed survival

during these critical months. Non-stalked fruit predominated during the late autumn, which increased larval survivorship and reduced the impact of low emergence rates on the size of the overwintering *E. vitisidaea* population.

Table 2 Number of *Epicephala vitisidaea* eggs and larval survivorship in two fruit types of *Breynia vitis-idaea* in summer and in late autumn

Season	Fruit type	Examined fruits	Number of larvae per fruit				Number of eggs	Number of living larvae	Larval survivorship (%)
			0	1	2	3			
Summer (Jul. – Aug.)	Non-stalked fruits	228	98	91	39	0	267	169	63.30
	Stalked fruits	495	378	92	25	0	520	142	27.31
	Sum	723	476	183	64	0	787	311	39.52
Late autumn (Nov.)	Non-stalked fruits	169	59	66	41	3	216	157	72.69
	Stalked fruits	37	26	11	0	0	37	11	29.73
	Sum	206	85	77	41	3	253	168	66.40

Larval survivorship = (the number of larvae + the number of exiting holes)/the number of eggs in fruits.

During the summer (June to August) we reared 80 *E. vitisidaea* larvae (57 from stalked fruits and 23 from non-stalked fruits) that developed into pupae. Forty-four of these pupae (31 from stalked fruits and 13 from non-stalked fruits) emerged as adults (emergence rate = 55%). *E. vitisidaea* overwinter as pupae for 4 months (Table 1). We observed 30 overwintering mature larvae (4 from stalked fruits and 26 from non-stalked fruits), only seven mature larvae (1 from stalked fruits and 6 from non-stalked fruits) pupated and emerged as adults in the spring (emergence rate = 23.3%). As such, emergence rates are lower for overwintering pupae. Larval survivorship and proportion of stalked fruits were significantly affected by an interaction of seasons and fruit types (Table 3). These studies have revealed a positive correlation between the proportion of non-stalked fruits and larval survivorship (Fig. 5). The high percentage of stalked fruits was related with the low larval survivorship in summer and the high percentage of non-stalked fruits was related with the high larval survivorship in late autumn.

Table 3 Two-way ANOVA for larval survivorship of *Epicephala vitisidaea* and fruit type of *Breynia vitis-idaea* in summer and late autumn

df	Larval survivorship		Proportion of non-stalked fruits		
	F	P	F	P	
Season (Se)	1	0.007	0.936	0.006	0.939
Stalked (St)	1	267.1	<0.01	141.6	<0.01
Se × St	1	10.99	<0.01	1372	<0.01

Significant P-values are in bold.

4 DISCUSSION AND CONCLUSION

Explaining the evolutionary stability of interspecific mutualism remains one of the major challenges in evolutionary biology (Sachs *et al.*, 2004;

West *et al.*, 2007; Kiers and Denison, 2008). Some figs limit the number of eggs laid by fig wasps by varying the length of the style, thereby ensuring seed production (Cook and Rasplus, 2003; Wang *et al.*, 2009). In the mutualism between *Glochidion acuminatum* plant and *Epicephala* moth, the proportion of aborted flowers progressively increased with higher egg load and thus increased the ovule damage (Goto *et al.*, 2010). Differing from the mechanism in the above mutualism, our study concerning the survivorship of *E. vitisidaea* larvae in stalked and non-stalked *B. vitis-idaea* fruits showed that the host plays a critical role in regulating the *Epicephala* population size by varying the stalk types that can prevent seed overconsumption. Survivorship rates of *E. vitisidaea* larvae that parasitized stalked fruits were lower than those that parasitized non-stalked fruits (Table 2). The stalk began to develop after the female moth laid the eggs, and *E. vitisidaea* individuals could not know whether the female flowers would develop into stalked fruits. As such, they could not lay their eggs selectively on non-stalked fruits to improve larval survivorship. The stalk developed before the *E. vitisidaea* eggs hatched (Fig. 3:D), lengthening the distance between the ovary and the egg. Larvae that hatched from eggs laid on the lower or middle part of the ovary of a stalked fruit had to climb up the stalk to access the ovaries. This climb consumed a great deal of energy and made the larvae more prone to be predated, thus increasing costs to individual larvae, making the feeding process more difficult, reducing larval survivorship, and decreasing seed consumption. Larvae hatched from eggs (Fig. 3:C) laid on the upper part of the ovary of stalked fruits or from eggs laid on non-stalked fruits did not have to climb a stalk and could directly feed on seeds. This decreased energy consumption and increased larval survivorship.

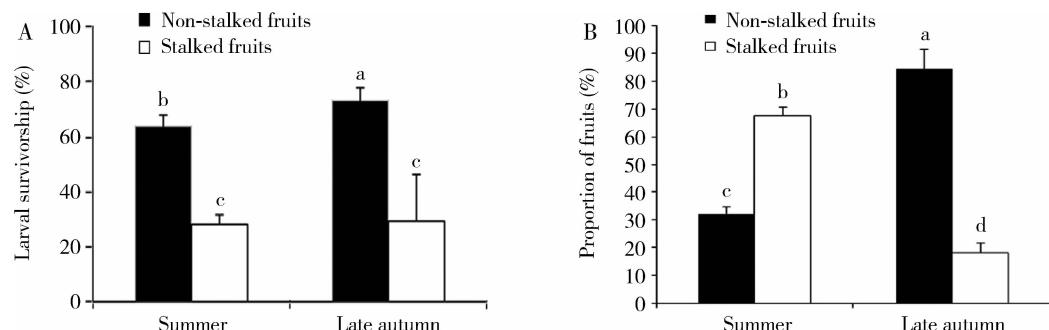


Fig. 5 Larval survivorship of *Epicephala vitisidaea* (A) and proportion of *Breynia vitis-idaea* fruits (B) in summer and late autumn. Bars are means \pm SE. Significant differences ($P < 0.05$) for each variable are indicated by different lowercase letters. Significant differences for each variable were compared using Duncan's multiple-range tests.

According to our field study, the proportion of non-stalked fruits decreased in summer and the larvae survivorship also decreased in summer (Fig. 5) (Table 2). The decreasing proportion of non-stalked fruits might attribute to the increasing moth population in summer, which resulted in more intensive competition of the common resources (flowers). This might lead to overexploitation by more eggs from the *Epicephala* moths, and might stimulate the *Breynia* plants to develop more stalk fruits to prevent seed overconsumption. The emergence rates of *E. vitisidaea* were lower in late autumn (23.3%) than in summer (55%). In addition, the proportion of non-stalked fruits of *B. vitis-idaea* was higher in late autumn (82.04%) than in summer (31.53%) (Table 2). Accordingly, larval survivorship was higher in late autumn (66.40%) than in summer (39.52%) (Table 2). The high proportion of non-stalked fruits in late autumn reduced the overwintering danger to *E. vitisidaea*, thereby decreasing the impact of low wintertime emergence rates on the *E. vitisidaea* population size and ensuring that enough *E. vitisidaea* adults emerged to pollinate *B. vitis-idaea* in the coming year. Emergence rates of *E. vitisidaea* increased in summer along with the proportion of stalked fruits. This decreased larval survivorship (39.52%) and prevented seed overconsumption by larvae.

We show here that *B. vitis-idaea* plants actively regulate the population size of *E. vitisidaea* moths in a season-specific manner, thereby creating a stable mutualistic relationship. This represents a new mechanism for facilitating mutualism.

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小叶黑面神与小叶头细蛾互惠共生 系统的种群调控机制

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摘要:【目的】探索小叶黑面神 *Breynia vitis-idaea* 对小叶头细蛾 *Epicephala vitisidaea* 种群数量的调控机制。【方法】跟踪记录小叶黑面神物候及头细蛾的生物学。解剖在小叶黑面神上访花头细蛾的外生殖器, 鉴定头细蛾种类。对不同时期小叶黑面神有梗和无梗的果实进行解剖, 统计果实内幼虫数量、果实表面孔的数量以及果实表面产卵疤数量, 计算头细蛾幼虫存活率。统计不同时期小叶黑面神有梗和无梗的果实的比例。【结果】在福建厦门小叶黑面神每年有 5 个花果期, 相应地, 为小叶黑面神传粉的头细蛾每年有 5 个生活世代。通过解剖, 该种头细蛾被鉴定为小叶头细蛾。一头小叶头细蛾幼虫需要消耗 2~4 粒种子才能发育成熟。小叶黑面神有两种不同形态的果实: 有梗和无梗。头细蛾幼虫在无梗果实内的存活率明显高于有梗果实, 并且晚秋时期头细蛾幼虫的存活率要高于夏季。小叶黑面神无梗果实的比例在晚秋(82.04%)要高于夏季(31.53%)。【结论】本研究揭示了维持互利共生体系稳定的机制。小叶黑面神能够通过果实基部果梗的有无来调节小叶头细蛾幼虫的存活率。小叶黑面神通过季节性的调节有梗果实的比率, 既有效避免了夏季种子被过度消耗的风险, 又提高了头细蛾在冬季的存活率。小叶黑面神这种自身调控机制对维持小叶黑面神与小叶头细蛾互惠共生系统的稳定性起到了至关重要的作用。

关键词: 小叶黑面神; 小叶头细蛾; 果梗; 幼虫存活率; 专性传粉互利共生; 调控机制

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